

Micromorphology of the elytral cuticle of beetles, with an emphasis on weevils (Coleoptera: Curculionoidea)



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ABSTRACT

The elytral cuticle of 40 beetle species, comprising 14 weevils (Curculionoidea) and 26 representatives of other taxa, is examined. All weevils and 18 other species have an endocuticle with prominent macrofibers, which corresponds to a modified pseudo-orthogonal cuticle. Angles between successive layers of macrofibers range between 30° and 90°, but are constantly less than 60° in weevils. In all Curculionoidea, as well as in one buprestid and one erolytid species exo- and endocuticle are densely interlocked. In the weevil *Sitophilus granarius*, transmission electron microscopy revealed vertical microfibrils extending from the exocuticle between the macrofibers of the underlying endocuticle. Vertical microfibrils connecting successive macrofiber layers of the endocuticle were observed in *S. granarius* and *Trigonopterus nasutus*.

Distinct cuticular characters are traced on a beetle phylogeny: the angles between unidirectional endocuticle layers; the presence and the shape of endocuticular macrofibers; and the interlocking of exo- and endocuticle. While character traits seem to be more or less randomly distributed among Coleoptera, the Curculionoidea have a uniform groundplan: The “weevil-specific” combination of characters includes 1) interlocking of exo- and endocuticle, 2) an endocuticle with distinct ovoid macrofibers embedded in a matrix and 3) comparatively small angles between successive endocuticular layers. Thus, phylogenetic constraints appear equally important to functional factors in the construction of the weevil elytron.

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1. Introduction

The transformation of delicate forewings into protective elytra might have been the evolutionary novelty that triggered the extraordinary diversification of the order Coleoptera (Crowson, 1981). The main function of the elytra is protection, thus their cuticle is usually thick and hardened. Among the ca. 350.000 described beetle species (Gullan and Cranston, 2014), both the elytra and the remaining cuticle are subject to considerable variation. The present study investigates the micromorphology of the elytral cuticle among different lineages and focuses on the weevils (Curculionoidea), a group comprising more than 60.000 described

species (Oberprieler et al., 2007). Many weevil species exhibit a very compact and hard exoskeleton (Crowson, 1981), especially in lineages that are flightless (Leschen and Beutel, 2014). The hyperdiverse genus *Trigonopterus* Fauvel is currently the subject of studies on its ecology (Riedel et al., 2010; Tänzler et al., 2012), biogeography (Tänzler et al., 2014) and functional morphology (van de Kamp et al., 2011; van de Kamp et al., 2014; van de Kamp et al., 2015a). Apparently, mechanical stability played a major role in the radiation of this group, because a more or less complex defence position (van de Kamp et al., 2014) belongs to its ground plan. Therefore, our initial aim was to examine the extremely hard cuticle of *Trigonopterus* for specific structures that may increase its stability and that differ from the general morphology of the weevil elytron. The final taxon selection was extended to cover all major groups of Coleoptera and to cover both winged and wingless species, especially of weevils.

Ontogenetically elytra are formed by epithelial folds of the larval thorax. Being duplications of the integument, elytra possess a thick

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dorsal and a thinner ventral cuticle connected by trabeculae and the hemolymph space between them (e.g. Chapman, 1998; Chen et al., 2007a,b; van de Kamp and Greven, 2010).

In general, the insect cuticle is a fibrous composite material consisting of a thin epicuticle almost without influence on the mechanic stability of the entire cuticle (Hepburn and Joffe, 1976) and a much thicker inner procuticle that consists of a protein matrix containing chitin microfibrils, usually arranged in layers. The procuticle of sclerites is subdivided into the outer sclerotized exocuticle and the inner unsclerotized endocuticle (e.g. Neville, 1975, 1993; 1998; Andersen et al., 1996; Andersen, 2010).

Microfibrils are either arranged helicoidally, i.e. one layer of parallel microfibrils is followed by another layer of slightly rotated orientation, or unidirectional, i.e. several layers of parallel microfibrils form a thicker preferred layer or “ply” (e.g. Neville, 1975, 1993). A cuticle of such a “plywood” appearance, in which the plies are separated by thin intervening zones of helicoidally arranged microfibrils is called “pseudo-orthogonal” (Neville and Luke, 1969). In a second type of the pseudo-orthogonal cuticle, parallel bundles of tightly packed microfibrils (and matrix) occur. The so-called “macrofibers” are aligned unidirectional, but change their direction in successive layers. These macrofibers correspond to the “Balken” in German literature (e.g. Kapzov, 1911; Kühnelt, 1928; Sprung, 1932; Scherf, 1954) and cuticles of this type are widespread in beetle elytra (Richards, 1951; Dennell, 1978). Macrofibers of a given horizontal layer appear to be connected by “intraply” cross linking fibers and those of successive layers by “interply” fibers in the elytra of *Pachnoda sinuata* examined by scanning electron microscopy (SEM) (Hepburn, 1972; Hepburn and Ball, 1973). However, transmission electron microscopy (TEM) of the cuticle of the weevil *Anthonomus grandis* showed neither interply/intraply fibers, nor helicoids between successive layers of macrofibers. Instead, spaces appear to be filled with a “fibrous matrix” probably of proteinaceous composition (Leopold et al., 1992: p. 52).

Structural features of the elytra of 40 beetle species representing 24 major families were examined by van de Kamp and Greven (2010) using semithin sections and SEM. Elytral cuticles showed considerable variations regarding overall thickness, thickness of the outer and inner cuticle and size of the hemolymph space. All species examined exhibited pseudo-orthogonal endocuticles. Five species representing five families showed a pure “ply-cuticle”, 33 species including all weevils had macrofiber cuticles and two species of different families had both plies and macrofibers.

Herein, we focus in particular on the boundary between exo- and endocuticle and some weevil-specific character traits. Furthermore, we use transmission electron microscopy images to illustrate intervening non-helicoid microfibrils between discrete macrofibers.

2. Material and methods

2.1. Specimens

Specimens belonging to 40 beetle species (Fig. 1) were taken from the collection of the State Museum of Natural History Karlsruhe (SMNK). The selection was restricted to species with hard cuticle, i.e. groups with soft cuticle, e.g. the Cantharidae were omitted. A focus was on phytophagous beetles, especially Curculionoidea; besides it was attempted to cover all major lineages of Coleoptera to allow comparison of observed structures. The selection of Curculionoidea covered small (e.g. *Trigonopterus nasutus*; body length ca. 3.4 mm) and large (*Gymnopholus subnacreus*; body length ca. 20 mm) species. Both winged and wingless species were examined.

2.2. Scanning electron microscopy

Dry pieces of the elytra were fractured vertically using fine forceps, the fragments coated with gold, and examined with a scanning electron microscope Zeiss LEO 1430VP at 20 kV. Fragments, which could be viewed directly from above and in which the direction of the lamellae was unaffected by surface sculpture or trabeculae were used to measure angles between successive plies.

2.3. Transmission electron microscopy

The weevils *T. nasutus*, *Sitophilus granarius* and *Cryptorhynchus lapathi* were examined by TEM. Small pieces of the elytra were stored in ethanol (96%), postfixed for 1 h in 2% osmium tetroxide in 0.1 M cacodylate buffer, washed, subjected to an ethanol dehydration series and embedded in epoxy resin (Spurr, 1969). Selected pieces of the elytra of *S. granarius* were treated with 10% KOH for 3 h before postfixation to remove proteins, a procedure not affecting fibril orientation in cuticles (Zelazny and Neville, 1972). Ultrathin sections were cut with a Reichert Om U3 microtome either using glass or diamond knives.

Sections were mounted on copper grids, stained with lead citrate (Venable and Coggeshall, 1965) and examined with a Hitachi H600 transmission electron microscope.

2.4. Light microscopy

Elytron samples were fixed and embedded as described in section 2.3 and cut with a C. Reichert Om U3 microtome using glass knives. Sections were stained with toluidine blue/borax solution, examined with an Olympus AH-2/Vanox T microscope and photographed with a Camedia Digital Camera C3030 Zoom.

3. Results

3.1. Scanning electron microscopy

Elytra of 14 species of Curculionoidea and 26 species representing 23 families of other beetles were examined by SEM and the following main characters of their elytral cuticle recorded: interlocking of exo- and endocuticle; appearance of macrofibers; size of macrofiber cross-section; angles between successive unidirectional endocuticular layers. Traced on a current phylogeny of beetles (Fig. 1) these traits appear more or less randomly distributed among Coleoptera. However, among Curculionoidea the combination of traits is remarkably stable.

All elytra show exo- and endocuticle. The exocuticles appear more or less homogeneous; microfibrils could not be sufficiently resolved. Except for five species, the endocuticle is composed of several layers of macrofibers aligned parallel to the surface. Macrofibers vary greatly in size, shape and arrangement (Fig. 1). Based on the appearance of the fractured surface, we distinguish endocuticles with frazzled (“fibrous”) macrofibers (Fig. 2 B), largely discrete macrofibers with a relative smooth fracture surface (Fig. 2 C), and macrofibers of variable size (Fig. 2 D). The species without endocuticular macrofibers show typical plies (Fig. 2 E). The angles between successive layers of macrofibers and plies can be divided into two groups: 1) angles ranging from 30° to 60° (e.g. *T. nasutus*, a species with smooth elytra; Fig. 2 F); 2) angles from >60° to 90° (e.g. *Tritoma bipustulata*; Fig. 2 G).

The endo- and exocuticles of all weevils are interlocked by exo- and endocuticular projections (Fig. 3 C–E). At least the exocuticular projections appear smooth in SEM. In *T. nasutus* they measure ca. 0.2 μm in thickness. Their counterparts in the endocuticle are macrofibers smaller than the underlying ones

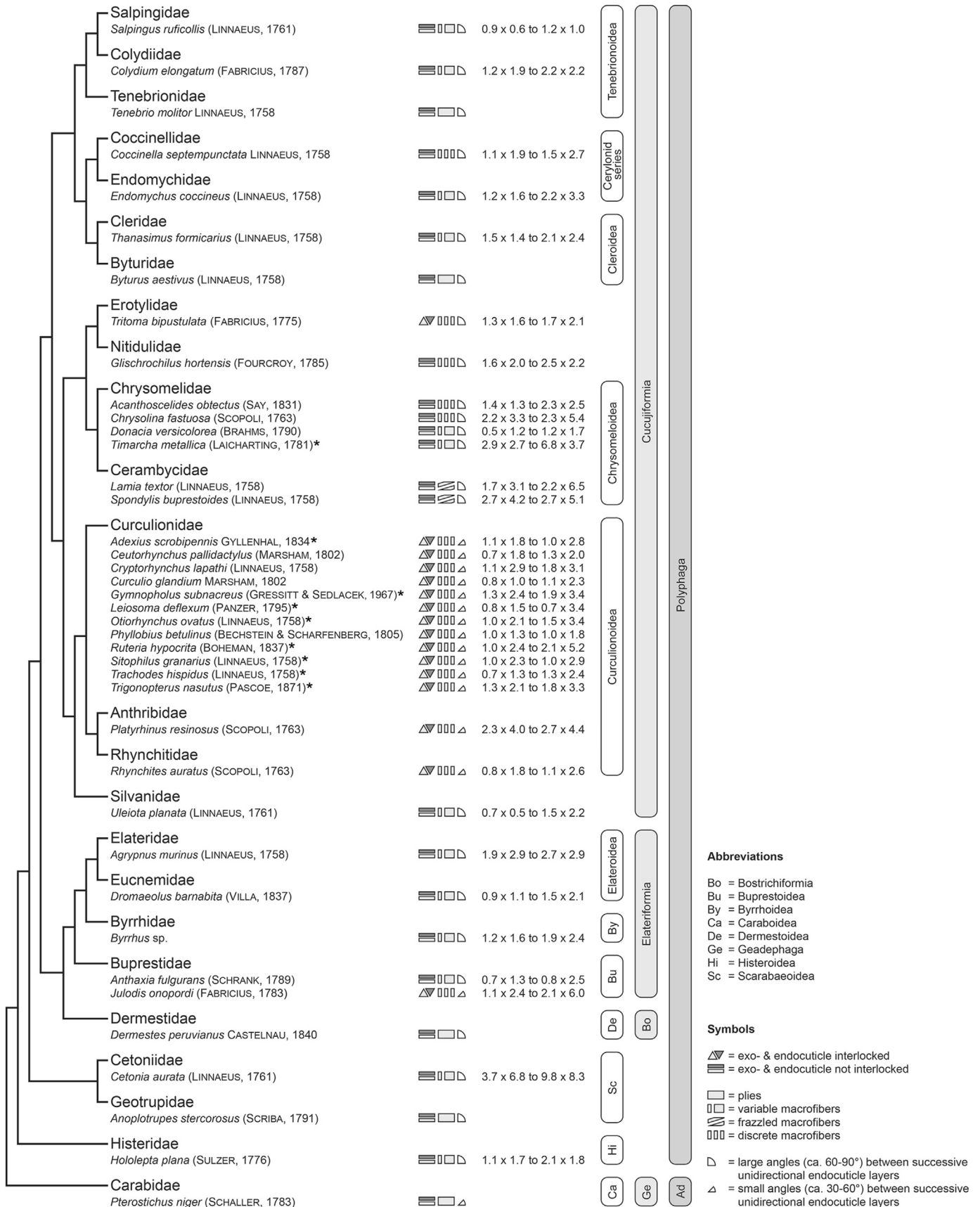


Fig. 1. Cladogram (according to Hunt et al., 2007) of the phylogenetic relations of Coleoptera families, from which species were studied, the species examined and morphological traits of their elytral cuticles seen in the SEM. Numbers indicate approximate sizes of macrofiber cross-sections in µm. * = flightless species.

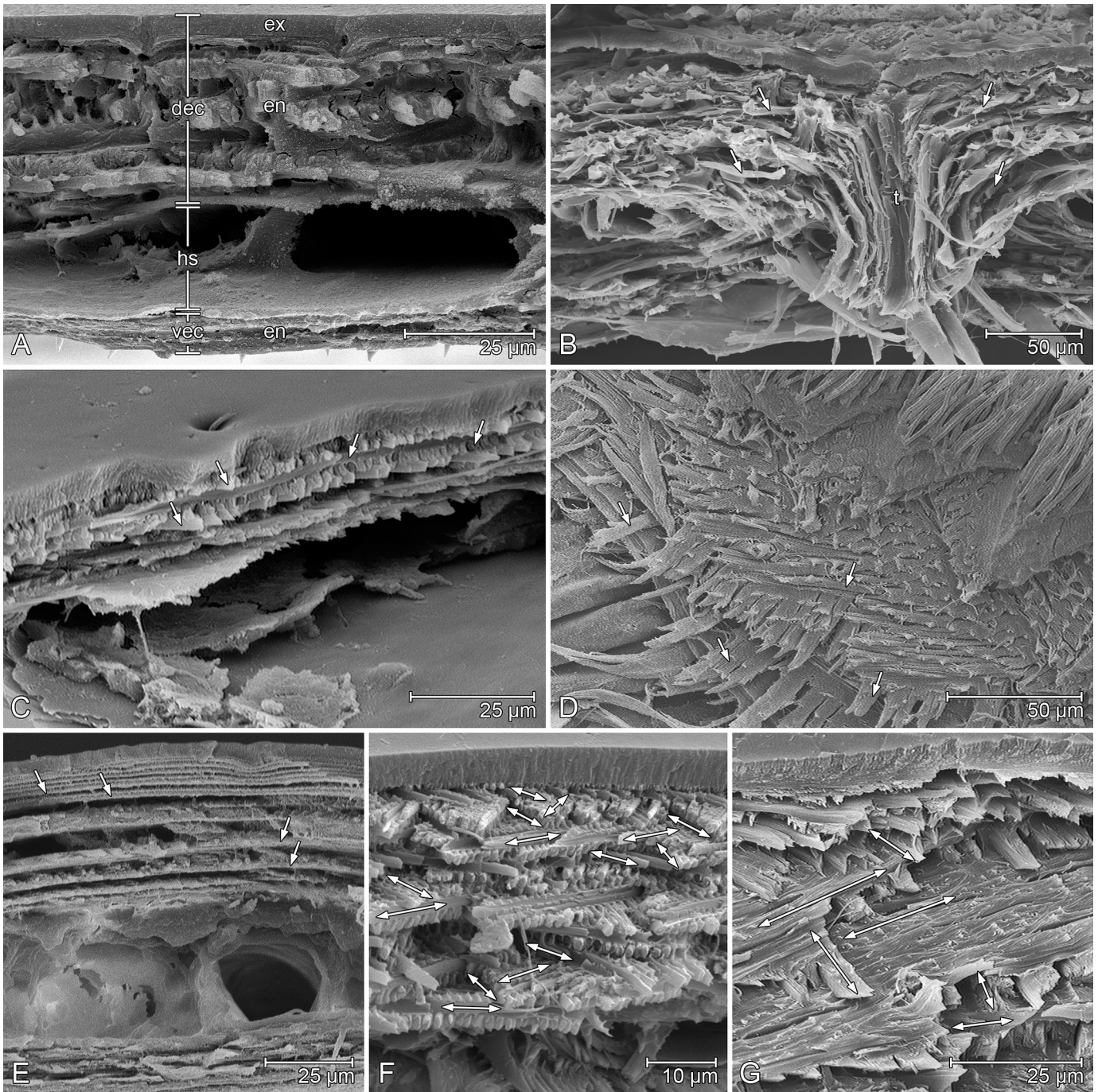


Fig. 2. Fractures of beetle elytra. SEM. **A.** *Chrysolina fastuosa*, overview showing the distinct cuticular portions. **B.** Frazzled macrofibers in the endocuticle of *Lamia textor*. Note the trabecle. **C.** Discrete macrofibers in the endocuticle of *Tritoma bipustulata*. **D.** Macrofibers of variable size in the endocuticle of *Cetonia aurata*. **E.** Plies in the endocuticle of *Pterostichus niger*. **F, G.** Angles between successive macrofiber layers are small as in *Trigonopterus nasutus* (**F**) or large as in *Timarcha metallica* (**G**). Macrofibers and plies are exemplary highlighted by single-headed arrows; orientation of macrofiber layers is indicated by double-headed arrows. dec = dorsal elytral cuticle; en = endocuticle; ex = exocuticle; hs = hemolymph space; t = trabecle; vec = ventral elytral cuticle.

(Fig. 3 E). Of the other taxa examined only *T. bipustulata* (Erotylidae) and *Julodis onopordi* (Buprestidae) show a comparable structure (Fig. 3 A,B, arrows).

3.2. Transmission electron microscopy

TEM images of the weevil endocuticle confirmed SEM data showing discrete macrofibers tightly packed and arranged in a regular pattern (Fig. 4 A–E). Most macrofibers have an ovoid cross section. In species with sculptured elytra, cross sections are

more irregular (Fig. 4 F). Macrofibers are embedded in a matrix and are thickest in the middle of the endocuticle and thinnest near the exocuticle and the epidermis. In the middle part of the endocuticle of *T. nasutus* five successive layers of macrofibers show a similar profile followed by a largely longitudinally cut macrofiber indicating small angles between the former (Fig. 5 A). The innermost endocuticle layers are smaller (arrow). Inside the macrofibers as well as in the surrounding matrix fibrils of ca. 5 nm diameter can be recognized. Some of these fibrils show a non-helical arrangement and extend vertically between

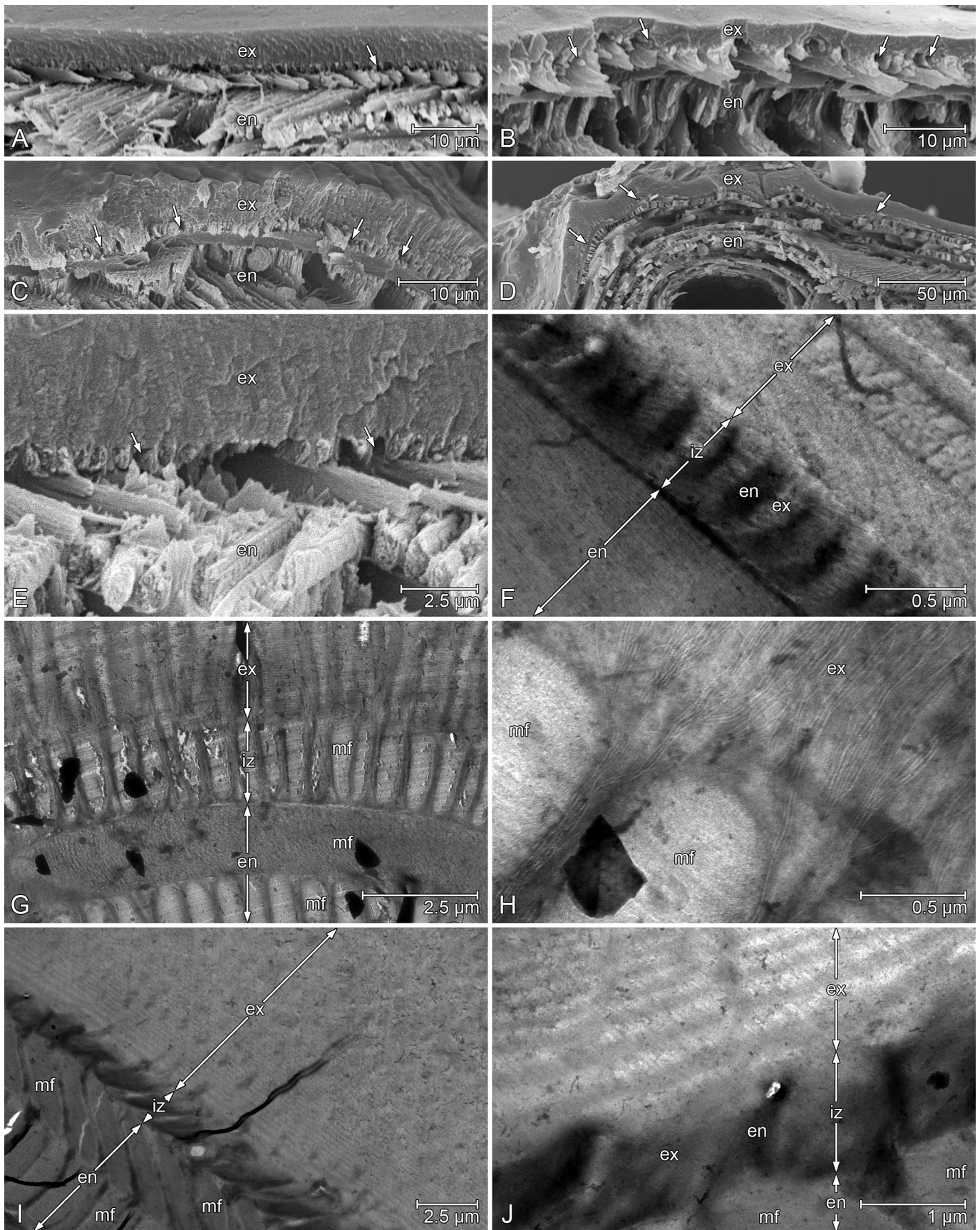


Fig. 3. Interlocking of the exo- and endocuticle (A–E: SEM; F–J: TEM). **A.** *Tritoma bipustulata* (Erotylidae). **B.** *Julodis onopordi* (Buprestidae). **C.** *Sitophilus granarius* (Curculionidae). **D.** *Cryptorhynchus lapathi* (Curculionidae). **E,F.** *Trigonopterus nasutus* (Curculionidae). **G,H.** *Sitophilus granarius*; note that fibers run perpendicular to the surface and extend between the macrofibers of the endocuticle. **I,J.** *Cryptorhynchus lapathi*; note the parallel arrangement of layers in the exocuticle. Interlocking is exemplarily highlighted by single-headed arrows. en = endocuticle; ex = exocuticle; iz = interlocking zone; mf = macrofiber.

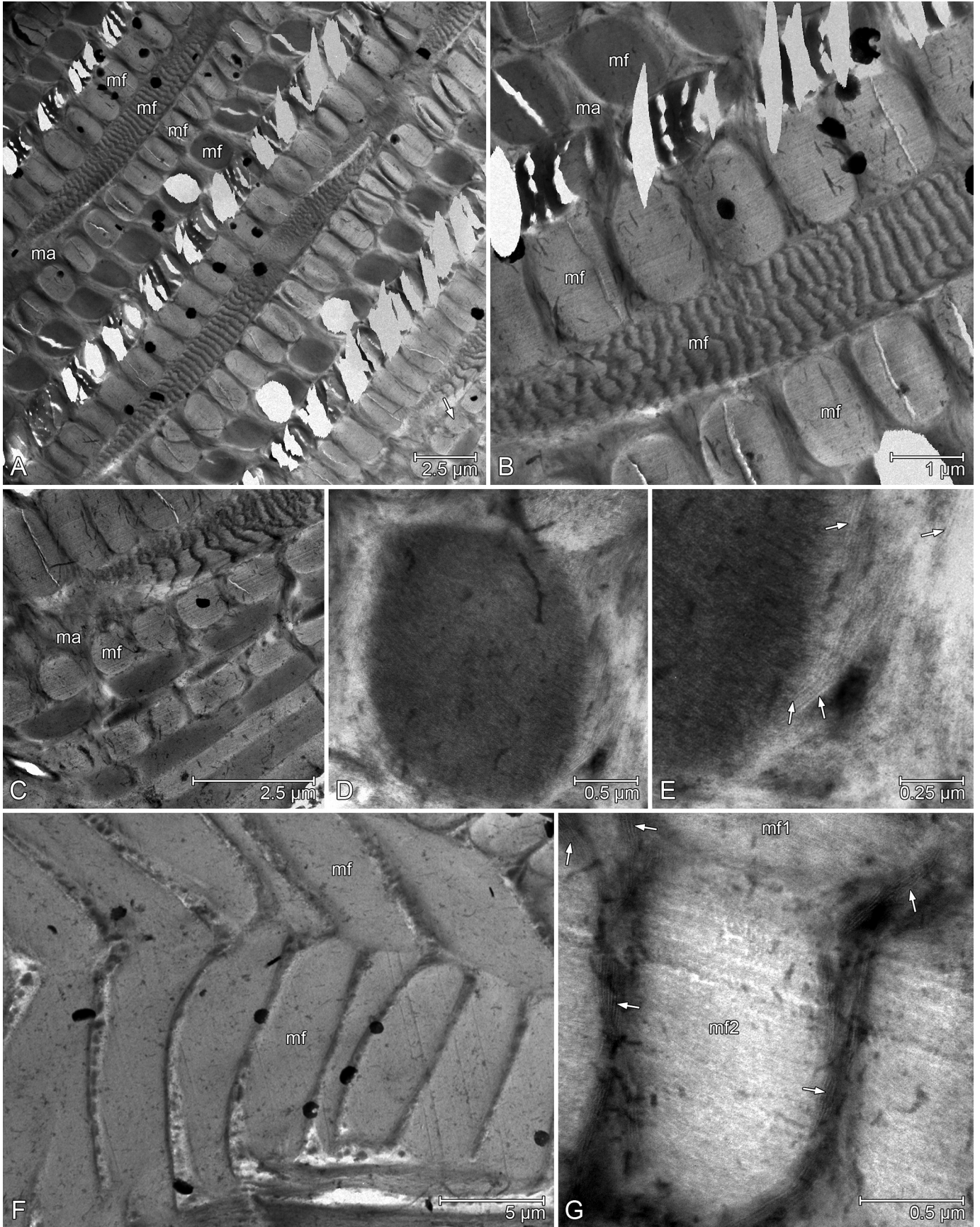


Fig. 4. TEM of the elytron endocuticle in weevils. **A.** *Trigonopterus nasutus*; endocuticle, overview; note the smaller macrofibers of the inner layers (arrow) and several layers of macrofibers of successive layers with similar profiles. **B.** *T. nasutus*; endocuticle; higher magnification; the black structures in the horizontal macrofiber are artefacts. **C.** *T. nasutus*; smaller macrofibers in the inner layers. **D.** *T. nasutus*; single macrofiber in matrix. **E.** section of **D**; detail of surrounding chitin microfibrils (arrows). **F.** *Cryptorhynchus lapathi*; irregular and arched macrofibers. **G.** *Sitophilus granarius*; microfibrils (arrows) connecting successive macrofiber layers (mf1 & mf2); sample treated with 10% KOH. mf = microfiber; ma = matrix.

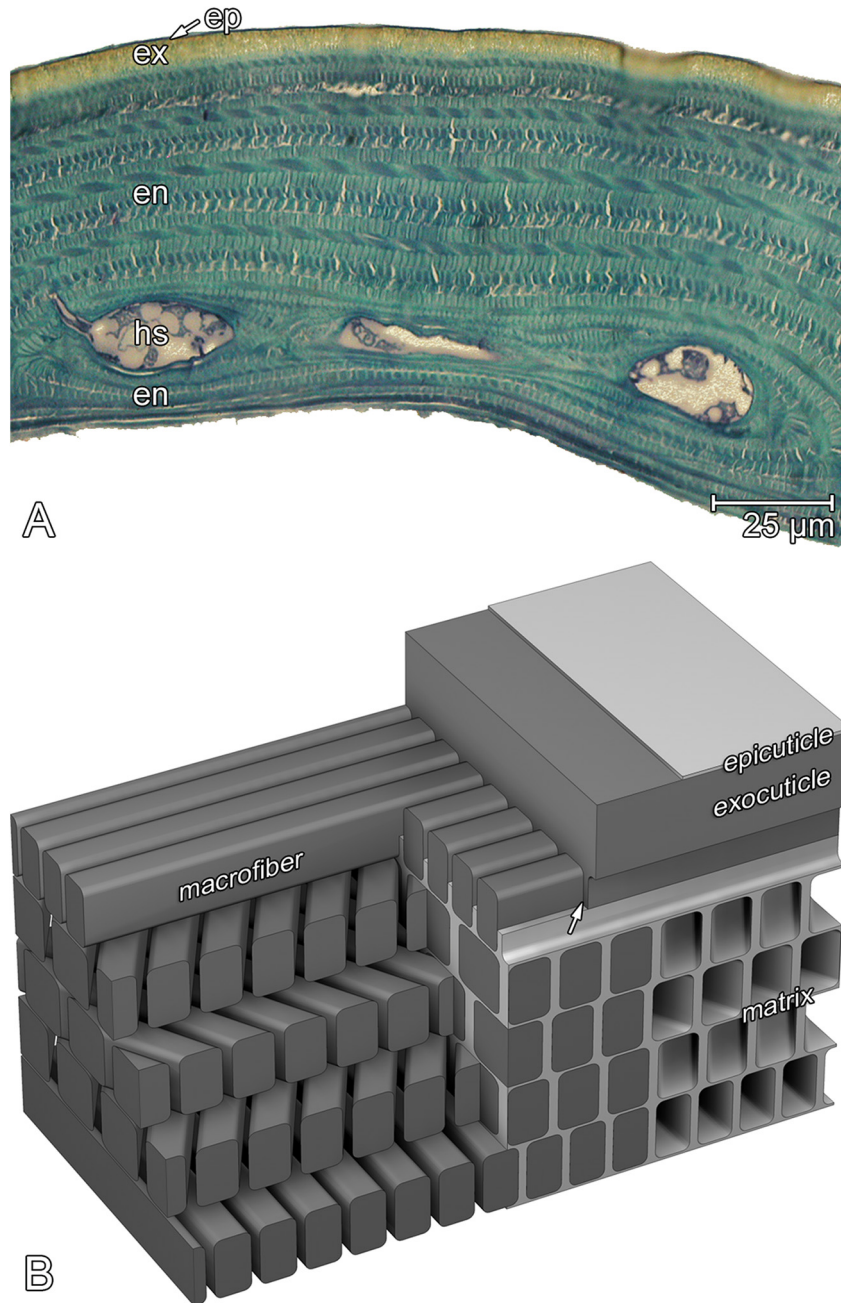


Fig. 5. Elytral cuticle of the weevil *Trigonopterus nasutus*. **A.** Semithin section (from van de Kamp and Greven, 2010). **B.** Diagram; note the interlocking between the uppermost macrofibers of the endocuticle with the exocuticle (arrow). en = endocuticle; ep = epicuticle; ex = exocuticle; hs = hemolymph space.

macrofibers of successive layers (Fig. 4 E,G). The interlocking between exo- and endocuticle appears to be variable. In *S. granarius* vertical fibrils with a diameter of ca. 5 nm extend from the exocuticle between the macrofibers of the endocuticle (Fig. 3 G,H). In *C. lapathi*, however, thin layers running parallel to the surface were observed (Fig. 3 I,J). The exo-/endocuticle boundary of *T. nasutus* resembles that of *C. lapathi* superficially, but the orientation of microfibrils is indistinct (Fig. 3 F).

4. Discussion

Pseudo-orthogonal endocuticle containing macrofibers is widespread among beetles (van de Kamp and Greven, 2010).

Therefore, it appears convenient to use the term “macrofiber cuticle” in addition to the term “pseudo-orthogonal cuticle” introduced by Neville and Luke (1969), because both represent the endpoints of a putative ply-macrofiber continuum. Between these endpoints a considerable variation exists in size and branching patterns of macrofibers, which may form reticulate layers, and the angles between successive layers of macrofibers (e.g. Reuter, 1937; Hepburn, 1972; Chen and Fan, 2004; Chen et al., 2007a; van de Kamp and Greven, 2010). The elytral cuticle of the weevils examined conforms to the general macrofiber pattern. Here, very discrete macrofibers with mostly ovoid profiles are embedded in a matrix as described by Leopold et al. (1992) for *A. grandis*. Regarding their overall appearance (at least in sections and

fractures) and the angles mentioned above constantly smaller than 60°, weevils adhere to a common groundplan. However, our estimation of angles may not reflect the real fluctuation range, which depends also on the body region (e.g. Dennell, 1978; Zelazny and Neville, 1972). It might even change between different layers in a given area of the endocuticle as shown in *T. nasutus* (van de Kamp and Greven, 2010). Angles may also vary within an individual and also might be generally smaller than 30° (Chen and Fan, 2004) and may occasionally reach > 60° in weevils (Leopold et al., 1992). TEM examination of the endocuticle of *S. granarius* and *T. nasutus* showed that both macrofibers and the matrix surrounding them contain fibrils, which appear to connect adjacent macrofibers horizontally and vertically, but are not arranged in a helicoid fashion. Their diameter of about 5 nm strongly suggests chitin microfibrils (Filshie, 1982: 5 nm; Neville et al., 1976: 2.3–3.5 nm; Vincent and Wegst, 2004: 3 nm).

An unusual character found in all weevils studied by SEM and in *T. bipustulata* (Erotylidae) and *J. onopordi* (Buprestidae) is the interlocking of endo- and exocuticle, which seem to establish a firm connection between the two cuticular zones. However, the mechanism of interlocking does not appear uniform even among weevils, as the structure of the exocuticle and their projections appears different in the two weevils examined by TEM. In the elytra of *S. granarius* fibrils resembling the ones observed in the endocuticle run perpendicular to the surface extending from the homogenous exocuticle between the macrofibers of the endocuticle, whereas in *C. lapathi* thin layers parallel to the surface were observed.

Arthropod cuticle structure has attracted considerable attention over the years and may lead to the development of cuticle-inspired materials in future (Chen and Fan, 2004; Gorb, 2011; Fernandez and Ingber, 2012). Its mechanical properties (stiffness, strength etc.) depend on numerous variables such as orientation and alignment of chitin microfibrils, protein and water content, compliance of layers, pigmentation, sclerotization, reinforcements in form of cuticular ribs, protuberances etc. (e.g. Hepburn and Ball, 1973; Hepburn and Chandler, 1976; Hepburn and Joffe, 1976; Wainwright et al., 1976; Hillerton et al., 1982; Chen and Fan, 2004; Vincent and Wegst, 2004; Barbakadze et al., 2006; Andersen, 2010; Dai and Yang, 2010; Lomakin et al., 2010; Yang et al., 2010; Klocke and Schmitz, 2011; Dirks and Taylor, 2012). Generally, cuticles are resistant to shear-and-crack propagation.

Interlocking of the hardened exocuticle with the more compliant endocuticle surely influences the cuticle's mechanical properties. This may also be the case in the unusual perpendicular orientation of microfibrils in the exocuticle as seen in *S. granarius*. A similar orientation has been documented by TEM from the head articulation cuticle in *Pachnoda marginata* (Barbakadze et al., 2006): here, the fibrils are oriented nearly perpendicular to the surface in the upper part of the exocuticle and parallel to the surface in the deeper layers of the exocuticle. In addition, the chitin microfibrils connecting the macrofibers vertically and horizontally in the endocuticle may contribute to the resistance against shear and crack as discussed for the “intraply” and “interply” fiber bundles connecting the macrofibers in the elytral cuticle of *P. sinuata* (Hepburn and Ball, 1973) and which probably represent bundles of microfibrils.

Fiber orientation heavily influences the material properties of composites and its effects in biological materials have been discussed in detail by Wainwright et al. (1976). Unidirectional composite materials are very anisotropic in plane, thus the stiffness of a single layer of macrofibers or plies varies considerably with the angle between the direction of the fibers and the line of action of the applied force. Cuticles with numerous unidirectional layers oriented in different directions can achieve high stiffness in more than one direction (Vincent and Wegst, 2004), as exemplified by

pseudo-orthogonal endocuticles. The weevil endocuticle with its small angles between the successive macrofiber layers may further reduce anisotropic effects.

As cuticle architecture is a trade-off between weight and stability, species with good flying abilities are expected to have a lighter but less robust exoskeleton, while in flightless beetles the cuticle can be thickened regardless the additional weight. Elytra of species with a high flight performance show a sophisticated lightweight construction, facilitated by an efficient distribution of trabeculae, which traverse a large hemolymph space between the dorsal and ventral cuticle (Chen et al., 2007a,b; van de Kamp et al., 2015a). Weevils are mostly slow-moving beetles. Many species are weak fliers or they are fully flightless. The Indo-Pacific genus *Trigonopterus* is known for its defensive strategy composed of a drop-off reflex and a state of death-feigning (thanatosis), which may be accompanied by mechanical blocking of the legs (van de Kamp et al., 2014). In this genus, the elytra are densely interlocked along the median suture and laterally with pterothorax and abdomen (van de Kamp et al., 2015a). A robust cuticle is crucial for their defensive strategy and indeed the elytra of *T. nasutus* (Figs. 2F, 3E,F, 4A–E and 5) are particularly thick (see also van de Kamp and Greven, 2010), but they do not possess any particular modifications from the elytral structure of other weevils.

It is remarkable that the 14 weevil species examined representing both apterous species and species capable of flight have some cuticular traits in common suggesting a greater role of phylogeny than functional constraints. A “weevil-specific combination”, e.g. distinct ovoid macrofibers embedded in a matrix, interlocking of exo- and endocuticle, and the comparatively small angle between successive layers are found equally in winged and wingless species. While the same combination of these characters was not found in the closely related Cucujiformia, it was observed in the buprestid *J. onopordi*, suggesting a convergent development.

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